Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2217–2221, March 1991
Biochemistry

Transforming function of proto-*ras* genes depends on heterologous promoters and is enhanced by specific point mutations

(upstream sequence/possible second untranslated exon/oncogene activation/recombination during transfection)

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Contributed by Peter H. Duesberg, December 13, 1990

ABSTRACT **Based on transfection into cells in culture or natural transduction into retroviruses, proto-*ras* genes seem to derive transforming function either from heterologous promoters or from point mutations. Here we ask how such different events could achieve the same results. To identify homologous regulatory elements, about 3 kilobases of rat DNA upstream of the first untranslated proto-Ha-*ras* exon was sequenced. Surprisingly, the sequence shares at −1858 a homology of 148 nucleotides with Harvey (Ha) sarcoma virus, 5′ of viral *ras*, signaling possibly a second untranslated proto-Ha-*ras* exon. In addition the sequence contains a perfect repeat of 25 CA dinucleotides at −2655. A retroviral promoter, even from upstream of the poly(CA), conferred transforming function on proto-Ha-*ras* and increased transcription >100-fold compared with that of unrearranged proto-*ras*. Point mutations were not necessary for transforming function of rat and human proto-Ha-*ras* genes with retroviral promoters but did enhance it >10-fold. A unifying hypothesis proposes that proto-*ras* genes depend on high expression from heterologous promoters or enhancers for transforming function, which is modulated by *ras* point mutations. The hypothesis makes two testable predictions. (*i*) Unrearranged proto-*ras* genes with point mutations, which occur in some cancers, have no transforming function. Indeed, tumors with mutated proto-*ras* genes, even those that also lack hypothetical tumor-suppressor genes, are indistinguishable from counterparts with normal proto-*ras* genes. (*ii*) Proto-*ras* genes in transfected cells derive transforming function from heterologous promoters or enhancers acquired via illegitimate recombination from vector DNAs and particularly from viral helper genes that must be cotransfected for transformation of primary cells. Indeed, expression of exogenous proto-*ras* genes in cells transformed by transfection is as high as for viral *ras* genes and is much higher than in the cells of origin.**

Harvey (Ha), BALB, Rasheed, and two other murine sarcoma viruses carry autonomous Ha-*ras* genes that cause tumors in animals and transform cells in culture (1–5). Kirsten sarcoma virus carries a structurally distinct but functionally very similar Ki-*ras* gene (1, 5). All viral *ras* (v-*ras*) genes differ from normal, cellular proto-*ras* genes in virus-specific promoters and virus-specific point mutations of their common *ras* coding region (2–9). Based on site-directed mutagenesis we (8, 9) and others (10) have shown that retroviral promoters are essential, and that point mutations are not necessary, for transforming function of v-*ras* genes.

Based on transfection into cells in culture, proto-*ras* genes appear to derive transforming function either from specific point mutations (11–13) or from heterologous promoters and enhancers (8–10, 14–16). For example, upon transfection, point-mutated proto-*ras* genes from some tumors transform the mouse NIH 3T3 line directly (11, 12) and transform primary rodent cells in conjunction with retroviral or DNA-viral helper genes (17–19). Alternatively, normal proto-*ras* genes linked to heterologous retroviral promoters also transform the NIH 3T3 mouse cell line (14, 16). Further, we (8, 9) and others (10) showed that normal rat proto-Ha-*ras* genes spliced into retrovirus vectors transform primary cells in culture and cause tumors in animals. Certain point mutations enhance the transforming function of transfected recombinant proto-*ras* genes >10-fold (8–10, 16).

Here we ask how such unrelated alterations of proto-*ras* genes as point mutation and replacement of native promoters and regulatory sequences by heterologous counterparts could each achieve the same transforming function (13). For this purpose the regulatory elements of rat proto-Ha-*ras* were sequenced‡ and functionally analyzed in a *ras*-containing retrovirus vector. We ask why point-mutated proto-*ras* genes depend on helper genes to transform primary cells *in vitro* (19) and are said to depend on inactivated suppressor genes (20–22) to cause tumors in animals or humans, whereas v-*ras* genes and normal proto-*ras* genes linked to retroviral promoters achieve the same results without helper genes. This appears paradoxical since all of these *ras* genes encode the same transforming protein.

RESULTS

Defining Untranscribed, Upstream Regulatory Elements of Rat Proto-Ha-*ras*. To identify noncoding regulatory elements of rat proto-Ha-*ras*, we planned to study a 2.5-kilobase (kb) *Hin*dIII-resistant rat DNA sequence that extends upstream of rat proto-Ha-*ras* in a λ phage clone prepared by Damante *et al.* (23). However, the presence of this fragment could not be confirmed in *Hin*dIII digests of the DNAs of Wistar, Fischer, and Buffalo rats (data not shown). Instead, each of these rats contained a 3.8-kb *Hin*dIII-resistant fragment that specifically hybridized with a 0.56-kb *Nae* I-resistant, exon 1-specific probe of the human proto-Ha-*ras* gene (24). This suggests that the upstream rat proto-Ha-*ras* region described by Damante *et al.* (23) was a cloning artifact. The 3.8-kb *Hin*dIII-resistant DNA fragment of rat proto-Ha-*ras* was also present in a λ phage carrying rat proto-Ha-*ras* DNA, termed NMU-8, that had been prepared by Notario and coworkers (25). About 3 kb from the 5′ *Hin*dIII site of the 3.8-kb fragment of phage NMU-8 was sequenced until an overlap of 200 nucleotides (nt) with the 5′ end of the rat proto-Ha-*ras* sequence of Damante *et al.* (23) was observed (Fig. 1). The sequence was numbered negatively from the 3′ splice donor site of proto-Ha-*ras* exon 1 (not shown in Fig. 1) because there is an uncertainty about the 5′ border of this exon (23, 26, 27). Comparisons of the upstream rat proto-Ha-*ras* sequence with Harvey sarcoma virus and human mouse proto-Ha-*ras* genes (Fig. 1) gave the following results.

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Abbreviations: nt, nucleotide(s); v-*ras*, viras *ras*.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M61016).

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                                        HaSV 917 ..... .......... .......... .......... .......... ..........  HaSV
.....t.... a......c.. g....t.... ..c...tg.. .t........ .a........ .......... .......... .......... .g.g.a...c  h
.......... .......... .......... ......ct.. .......... .......... .......... .......... t......... ......c...  m
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FIG. 1. Nucleotide sequence of rat DNA from proto-Ha-*ras* exon 1 to a *Hin*dIII site 3 kb upstream. The sequence was derived from a plasmid clone (see text) by Lark Sequencing Technologies, Houston, TX. The sequence is compared with that of Harvey sarcoma virus (HaSV) (5), mouse (m) proto-*ras* (26), and human (h) proto-Ha-*ras* (24). Dots signal identity and lines the absence of nucleotides. Possible control elements [TATA, GC, and CCAAT boxes and a poly(CA) sequence] are capitalized (see text).

(*i*) Except for one extra G at position −157 the 200-nt overlap from the 3′ end of our sequence was identical with the 5′ end of that published by Damante *et al.* (23).

(*ii*) Upstream of the conserved splice donor coordinate of exon 1, rat proto-Ha-*ras* shares a closely related sequence of ≈300 nt with human proto-Ha-*ras* (24) (partially shown in Fig. 1) and essentially all of the 478 nt from the splice donor coordinate of mouse proto-Ha-*ras* that have been sequenced (26) (Fig. 1). The conserved sequences include the proposed 5′ G+C rich-("GC") promoter of rat proto-*ras* (23), which maps at position −148, and a CCAAT box at −212 thought to bind transcription factors (23, 26).

(*iii*) The 3-kb rat sequence does not contain any large continuous coding sequence in any of the three possible reading frames.

(*iv*) The sequence contains multiple homopolymeric T and A stretches and, at −2655, a perfect poly(CA) sequence of 25 CA dinucleotides. About 50,000 poly(CA) repeats are present in all eukaryotes in regions flanking known human and animal genes (28). Such sequences have been proposed as transcriptional silencers, as in the case of the rat prolactin gene (29).

(*v*) Surprisingly, homology with Harvey sarcoma virus extends, after a gap from near the 5′ end of proto-Ha-*ras* exon 1 at positions −117 to −1710, for about 148 nt to −1858, signaling possibly a sixth proto-Ha-*ras* exon. The homology with the Harvey virus is preceded by a canonical TATA box at −1996 and a CCAAT box at −2016. Moreover, the estimated size of proto-Ha-*ras* mRNA including this hypothetical exon would be 1.4 kb, consistent with measurements by us (see Fig. 3) and others (11). A recently discovered relative of the proto-Ha-*ras* genes with six exons, termed R-*ras*, may be a precedent (30).

It appears that the coding region of rat proto-Ha-*ras* is preceded by either one or two untranslated exons and regulatory elements including a poly(CA) sequence.

Transforming Function of Recombinant Viruses with Normal Rat Proto-Ha-*ras* Including Untranscribed Upstream Sequences. To determine whether native upstream sequences

could suppress rat proto-Ha-*ras* transforming function derived from a retroviral promoter, a retrovirus was constructed that contains 6.2 kb of rat DNA from the *Hin*dIII site that maps 3.8 kb upstream of the coding region, at −3021 in Fig. 1, to an *Xba* I site that maps at the end of the last proto-Ha-*ras* exon (7) (Fig. 2). The retroviral promoter is encoded in the long terminal repeat of the provirus (5). The recombinant provirus, pA6.2-pras (Fig. 2), was transfected together with cloned helper Moloney provirus, pZAP, into mouse C3H/10T½ cells as described (8, 9). About 2–3 weeks later 6–10 foci per μg of pA6.2-pras DNA were obtained. Similar yields in terms of foci per microgram of proviral DNA were obtained upon transfection of C3H/10T½ cells with provirus clones, pApras 1-X (8) (Fig. 2) and pBpras (9), that carried the four coding exons of rat proto-Ha-*ras* in Harvey and BALB sarcoma virus-derived vectors. By contrast, >10-fold more foci were obtained when otherwise identical proviruses with point-mutated proto-Ha-*ras* genes were analyzed (8, 9).

To determine whether the recombinant virus generated from the pA6.2-pras proviral construct had retained the complete upstream sequence of the rat proto-Ha-*ras* gene, the viral RNAs of three focus-derived virus stocks were analyzed. For this purpose, foci of C3H/10T½ cells transformed by pA6.2-pras were grown into mass cultures and virus stocks were harvested from the growth media. Subsequently, intracellular viral RNA of C3H/10T½ cells infected by three clonal A6.2-pras virus stocks and of a Apras 1-X virus control were analyzed by 1% agarose/2 M formaldehyde electrophoresis (31) and hybridized with an Ha-*ras*-specific ^{32}P-labeled DNA probe derived from BALB sarcoma provirus (Fig. 3). The RNAs of two A6.2-pras virus clones, nos. 1 and 2 (lanes 2 and 3 in Fig. 3 *A* and *B*) each included a major species of ≈7.5 kb, the predicted size of a full-length RNA transcript of pA6.2-pras (Fig. 2). In addition, they each included a 4-kb and about 2.2-kb subgenomic *ras*-specific RNA. The 4-kb *ras* RNA roughly corresponds to a transcript from which all proto-Ha-*ras* introns, including the one between the possible untranslated exon X? and the untranslated exon X1 are spliced (Fig. 2). The 2.2-kb *ras* RNA corresponds to a transcript that is initiated from the proto-Ha-*ras* promoter preceding either exon X? or exon X1. Further work is necessary to validate these assignments. The RNA of pAras 1-X virus measured ≈3 kb (Fig. 3, lane 4), as expected for a fully spliced transcript of pApras 1-X (Fig. 2). The RNA of A6.2-pras virus clone 3 measured only 2.8 kb (Fig. 3, lane 5). This small size is consistent with a spontaneous deletion of nearly all noncoding sequences of pA6.2-pras (Fig. 2), retaining only a fully spliced *ras* coding sequence and some untranslated sequences. Fig. 3*C*, lanes 2–5, shows the 8-kb genomic and the 3-kb subgenomic *env* mRNA of Moloney helper virus (5) after hybridization of a Moloney virus-specific DNA probe to the blot shown in Fig. 3 *A* and *B*.

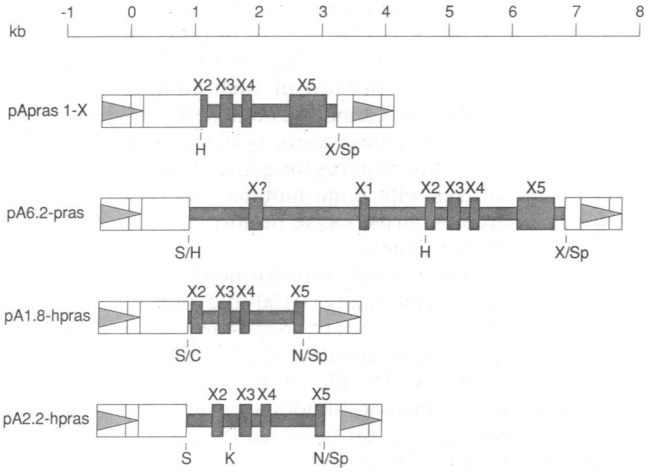

FIG. 2. Genetic structure of three recombinant proviruses carrying rat and human proto-Ha-*ras* genes. pApras 1-X is a modified Harvey sarcoma provirus carrying between its 5′ *Hin*dIII (H) and 3′ *Spe* I (Sp) sites the four coding exons flanked by the *Hin*dIII (H) and *Xba* I (X) sites of rat proto-Ha-*ras* (8). pA6.2-pras was derived from pApras 1-X by inserting, between the viral *Sst* II (S) site and the proto-Ha-*ras Hin*dIII site, the 3.8-kb *Hin*dIII-flanked rat proto-Ha-*ras* sequence that maps 5′ of the four coding exons in the rat genome (see text). pApras 1-X (8) was modified by deleting an *Eco*RI- and Mst II-flanked region of Harvey sarcoma virus that carries a *Hin*dIII site located 5′ of the provirus shown and by converting the viral *Sst* II site to a *Hin*dIII site with a synthetic linker. pA1.8-hpras carries a 1.8-kb human proto-Ha-*ras* sequence that extends from a *Cel* II (C) site 5′ of the second exon to a *Not* I (N) site 3′ of the translation stop codon in exon 5 (24) in the same vector as pA6.2-pras. The *Sst* II and *Spe* I sites of our Harvey vector were joined with a synthetic polylinker including *Cel* II- and *Not* I-compatible sites to construct this and the following recombinant. pA2.2-hpras carries a 2.2-kb *Sst* II- and Not I-flanked human proto-Ha-*ras* sequence including exons 2–5. K marks a *Kpn* I site used to exchange normal and mutated exon 2 (see text). The boxes with a shaded arrow are retroviral long terminal repeats that include the retroviral promoter and the transcription start signal (5), marked by a vertical line. The adjacent white boxes are virus-derived noncoding regions (5, 8). Rat and human proto-Ha-*ras* exons are represented by shaded boxes (X1–X5) and a possible exon is marked (X?). The proto-Ha-*ras* introns and rat noncoding regions are marked by narrow shaded boxes.

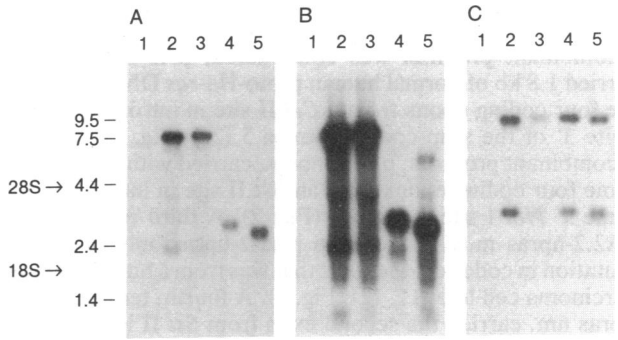

FIG. 3. Intracellular RNAs of recombinant retroviruses carrying proto-Ha-*ras* genes. About 5 μg of poly(A)-tagged RNA of mouse C3H/10T½ cells transformed by one of three focus-cloned A6.2-pras virus stocks, numbered 1, 2, and 3 (lanes 2, 3, and 5), derived from recombinant provirus pA6.2-pras (Fig. 2), or by Apras 1-X virus (lane 4), derived from recombinant provirus pApras 1-X (Fig. 2), and of uninfected C3H/10T½ cells (lane 1) was electrophoresed in buffered 1% agarose containing 2 M formaldehyde (31). The proto-Ha-*ras*-carrying viruses were propagated with helper Moloney retrovirus as described (8, 9). Total cellular RNA was extracted from about 10^8 cells by the guanidinium thiocyanate method, pelleted through 6 ml of a saturated solution of CsCl by centrifugation in a Beckman SW27 rotor at 25,000 rpm and 5°C, and passed over an oligo(dT) column to select poly(A)-tagged RNA species (31). After electrophoresis, the RNA was degraded in the gel by incubation at room temperature with 50 mM NaOH for 45 min, and after neutralization with 1 M NH_4OAc it was blotted onto a nitrocellulose filter (31). (*A* and *B*) The filter was hybridized with a 0.6-kb BALB sarcoma provirus-derived, *Hin*dIII- and *Bam*HI-flanked *ras* DNA (2) that was labeled to a specific activity of ≈10^9 cpm/μg by using [α-^{32}P]dCTP and commercial DNA polymerase and oligonucleotide primers (Amersham, RPN 1601). Hybridization was in 50% formamide containing 10% dextran sulfate, 5× SSC (SSC is 0.015 M sodium citrate/0.15 M NaCl), and other supplements as described (31). After hybridization the filter was washed twice for 20 min at 50°C in 0.2× SSC and autoradiographed for 2 hr (*A*) and 24 hr (*B*). (*C*) The filter was placed in 0.05% SDS at pH 7 for 1 min at 100°C to remove the ^{32}P-labeled *ras* DNA and then hybridized with a synthetic 41-base ^{32}P-labeled DNA probe specific for Moloney virus (obtained from M. Pech and S. A. Aaronson, National Cancer Institute) and autoradiographed as above for 3 hr. Ordinate indicates the sizes of commercial RNA standards (BRL) in kilobases and the location of the 28S and 18S rRNAs.

It follows that the retroviral promoter predominates over possible transcriptional suppressor elements like the poly(CA) sequence of the upstream proto-Ha-*ras* sequence. Suppressor elements encoded in upstream untranscribed sequences of the proto-*sis* gene are also overridden by a retroviral promoter (32). By contrast, a retroviral promoter failed to override upstream suppressor elements of chicken proto-*myc* (33).

The enhancement of transcriptional activity imparted to rat proto-Ha-*ras* by a retroviral promoter can be compared with the activity of the native proto-Ha-*ras* in the same cell by comparing in Fig. 3 *A* and *B* the amounts of viral *ras* RNAs (lanes 2–5) with the amounts of endogenous 1.2- to 1.4-kb proto-Ha-*ras* mRNAs of C3H/10T½ cells (lanes 1–5). In Fig. 3*A* the endogenous proto-Ha-*ras* mRNA is completely undetectable. Upon extended autoradiography, it becomes visible in virus-infected cells (lanes 2–5) and barely visible in the same amount of RNA from untransformed and uninfected C3H/10T½ cells (Fig. 3*B*, lane 1). It is estimated that the retroviral promoter enhances expression of proto-Ha-*ras* at least 100-fold compared with native proto-Ha-*ras*. Large differences between the expression of transforming proto-*ras* genes linked to retroviral or DNA-viral promoters and unrearranged, normal proto-*ras* genes have also been observed by others (10, 14, 15).

Transforming Function of a Recombinant Virus with Normal Human Proto-*ras*. Next we determined whether the human proto-Ha-*ras* in our Harvey virus-derived vector depended on point mutations for transforming function, as one previous study had reported (6). For this purpose, a recombinant provirus was constructed, pA1.8-hpras, that carried 1.8 kb of normal human proto-Ha-*ras* DNA including the four coding exons from a *Cel* II site in intron 1 to a *Not* I-site 3′ of the stop codon in exon 5 (24) (Fig. 2). Another recombinant provirus, pA2.2-hpras, carried within 2.2 kb the same four coding exons from an *Sst* II site in intron 1 to the same 3′ *Not* I site in exon 5 (Fig. 2). A third recombinant, pA2.2-hpras m, differed from pA2.2-hpras only in a point mutation in codon 12 of exon 2 that was from a human bladder carcinoma cell line (11, 12) (Fig. 2). A fourth, termed pA2.2-hpras nm, carried the second exon from *Sst* II in intron 1 to *Kpn* I in intron 2 (Fig. 2) derived from normal human proto-Ha-*ras* and the remainder from the proto-Ha-*ras* of the human bladder carcinoma cell line.

The recombinant proviruses were transfected together with helper Moloney provirus pZAP into C3H/10T½ cells as described above. Within 2–3 weeks about 4–6 foci of transformed cells appeared per μg of pA1.8-hpras and pA2.2-hpras nm DNA, about 1–3 per μg of pA2.2-hpras DNA, and 50–100 per μg of pA2.2-hpras m DNA with the point mutation. The 10-fold higher yield of foci per microgram of mutant proto-Ha-*ras* provirus compared with proviruses with normal proto-Ha-*ras* DNA confirmed and extended previous results in which point mutation was shown to enhance >10-fold the focus yield of recombinant proviruses with rat (8–10) and human (16) proto-Ha-*ras* DNAs. It is concluded that human proto-Ha-*ras* linked to a retroviral promoter does not depend on point mutations for transforming function.

DISCUSSION

In the light of our results and those from others, we propose a unifying hypothesis which holds that proto-*ras* genes depend on heterologous promoters or enhancers for transforming function and that point mutations are not sufficient for transforming function. The strength of the heterologous promoters and specific point mutations would each modulate the efficiency of transforming function. For example, among human proto-Ha-*ras* genes transcriptionally activated by a retroviral promoter, those with a point mutation generated ≈10 times more foci than those without. However, without heterologous promoters *ras* genes with or without point mutations have no transforming function. This hypothesis confirms and extends the recombinant cancer gene hypothesis which holds that normal cellular protooncogenes derive transforming function from heterologous promoters and coding elements acquired by illegitimate recombination with viral or cellular genes (8, 34, 35).

Our hypothesis makes two testable predictions: (*i*) unrearranged proto-*ras* genes with point mutations, which occur in some tumors, have no transforming function, and (*ii*) transforming function of proto-*ras* genes from tumors observed upon transfection *in vitro* reflects heterologous promoters or enhancers acquired via illegitimate recombination.

The following evidence supports these predictions. (*i*) Point-mutated proto-*ras* genes are found in only a minority of spontaneous tumors (5, 36) and do not distinguish these tumors from counterparts without point mutation (55). For example, only ≈40% of otherwise indistinguishable colon carcinomas contain point-mutated proto-Ki-*ras* genes (37, 38). Since point-mutated proto-Ki-*ras* genes do not transform primary cells, it was recently postulated that they depend on the loss or the inactivation of at least four tumor-suppressor genes for transforming function (20–22). However, only 35% of otherwise indistinguishable colon cancers lack a presumed suppressor gene, *APC* (adenomatous polyposis coli), located on chromosome 5, and 70% lack the presumed suppressor gene encoding protein p53, located on chromosome 17, and 70% lack the presumed suppressor gene *DCC* (deleted in colon cancer) located on chromosome 18 (20–22). Thus, a mere 7% (0.40 × 0.35 × 0.70 × 0.70) of otherwise indistinguishable carcinomas contain four of the five postulated cooperating oncogenes. In view of such poor correlations, point-mutated *ras* genes cannot be considered necessary for transforming function unless there is direct functional evidence. Moreover, the suppressor-gene hypothesis fails to explain how viruses with point-mutated *ras* genes, such as the Kirsten sarcoma virus, cause tumors in animals that do not lack suppressor genes.

(*ii*) Proto-*ras* genes in cells transformed by transfection or infection are transcriptionally ≈100-fold more active than unrearranged proto-*ras* genes of normal and tumor cells (Fig. 3). Indeed, their activity equals that of v-*ras* genes in virus-transformed cells (14, 15, 18, 39–42). By contrast, point-mutated proto-*ras* genes in tumors are transcriptionally not more active than normal proto-*ras* genes in otherwise indistinguishable tumors (38) or in nonmalignant control tissues (26, 43). Thus exogenous proto-*ras* genes, in cells transformed by transfection, must derive high transcriptional activity from heterologous promoters and enhancers. These promoters could derive from plasmid, carrier, or recipient cell DNA, and above all, from heterologous viral helper genes cotransfected for transformation of primary cells (17, 19). The apparent requirement for viral helper genes to transform a primary cell (17–19) would reflect merely the requirement for a source of potent heterologous promoters or enhancers equivalent to those of v-*ras* genes, which are capable of transforming primary cells directly (5, 34). Such promoters or enhancers would be linked to proto-*ras* genes by illegitimate recombination during transfection (44, 45), generating transcriptionally activated recombinants that are functionally similar to the proto-Ha-*ras* recombinant viruses described here. This proposal resolves the apparent paradox that proto-*ras* genes with point mutations depend on viral helper genes to transform primary cells upon transfection, whereas the same genes artificially linked to retroviral and DNA viral promoters (see above and refs. 8–10, 15, and 16), as well as authentic v-*ras* genes, transform primary cells without helper genes, although all of these genes encode the same transforming protein. Hence the relevance of the transfection assay into NIH 3T3 cells would be limited to the detection of point mutations, because such mutations en-

hance transforming function of proto-*ras* genes with random heterologous promoters sufficiently to detect them against the background of spontaneous 3T3 cell transformation.

The following observations are consistent with this view. (*a*) Among transfected cells with similar copy numbers of exogenous proto-*ras* genes, only those that express high levels of *ras* mRNA are morphologically transformed (40). (*b*) Some transformants express *ras* mRNAs that are larger than normal and thus are obvious recombinant *ras* mRNAs (40, 41, 46). (*c*) Deletion analyses show that the promoters and enhancers, rather than the coding regions of viral helper genes, are critical to complement cotransfected proto-Ha-*ras* genes (19). For example, deletion mutants of proto-*myc* genes transcriptionally activated by retroviral promoters, which are totally inactive as autonomous genes, are fully active as helper genes of point-mutated proto-*ras* (42). (*d*) Only proto-*myc* genes with heterologous retroviral promoters function as helper genes for transformation by proto-*ras* (40, 41). (*e*) Resident proto-*ras* genes of cells transfected by helper genes are not "activated" (19), as would be expected from complementation mediated by cooperating gene products. It appears that the transfection assay does not measure transforming function of native proto-*ras* genes in tumors but generates artifacts that derive transforming function from heterologous promoters or enhancers.

Thus, neither are there transformation-specific markers that distinguish tumors with point-mutated proto-*ras* genes from tumors with normal proto-*ras* genes, nor is there direct functional evidence for the hypothesis that unrearranged proto-*ras* genes derive transforming function from point mutations. The hypothesis has been questioned by others because of poor correlations with point-mutated proto-*ras* genes among otherwise indistinguishable tumors, because proto-*ras* genes from tumors fail to transform primary cells, and because point-mutated proto-*ras* genes appeared only after the tumor had metastasized (47–52). It is consistent with this view that proto-*ras* genes with point mutations are observed in benign, reversible hyperplasias (43, 53) and in normal transgenic mice (54), while retroviral *ras* genes encoding identical proteins are inevitably oncogenic.

Note Added in Proof. As predicted above, the human proto-Ha-*ras* gene with a point mutation in codon 12 (11, 12) transformed primary rat cells upon cotransfection with the promoter of Harvey sarcoma virus (Steve Clark, A.K.C., and P.H.D.).

We thank Harry Rubin, Bryan Ellison, and G. Steven Martin for reviews, Stuart Aaronson and Matthias Kraus of the National Cancer Institute, Bethesda, MD, for advice and support during a sabbatical stay of P.H.D., Vicente Notario of Georgetown University for phage NMU-8, and Allan Balmain for critical information. This study was supported by Outstanding Investigator Grant 5-R35-CA29915-06 from the National Cancer Institute.

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